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Glatiramer acetate treatment does not modify the clinical course of (NZB × BXSB)F1 lupus murine model

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Abstract

Glatiramer acetate (GA, copolymer-1, Copaxone®), a therapy approved for treatment of multiple sclerosis (MS), prevents and reverses experimental autoimmune encephalomyelitis, the animal model of MS. In central nervous system autoimmune disease, GA is thought to act through modulation of antigen-presenting cells, such as monocytes, mediating an antigen-independent T_h2 shift and development of FoxP3+ regulatory T cells. Recent reports indicate that GA may also be effective in models of other autoimmune diseases such as uveoretinitis, inflammatory bowel disease and graft rejection. To date, the potential effect of GA in lupus animal models has not been described. (NZB × BXSB)F1, male mice bearing *Y-linked autoimmune acceleration* , is a lupus-prone mouse model which is associated with a monocytosis accelerating disease progression. These mice were treated with GA before disease onset until death and both mortality rate and biological parameters were assessed to investigate whether GA may be beneficial in this spontaneous model of systemic lupus erythematosus. GA exerted no beneficial effect on the median survival after up to 7 months of treatment. Humoral and cellular parameters used as markers for lupus progression, such as anti-chromatin, anti-double-stranded DNA and anti-erythrocytes antibodies, hematocrit and monocytosis, were similarly unchanged. Our study demonstrates that GA has no significant effect on the progression of the (NZB × BXSB)F1 lupus-prone animal model. These results reinforce the hypothesis that GA may exert its beneficial effect in some specific autoimmune diseases only.

Introduction

Glatiramer acetate (GA) (Copaxone®) is a copolymer of 40–100 residues which is randomly composed of the four amino acids glutamate, lysine, alanine and tyrosine in a defined molar ratio (1). Initially developed to mimic a major component of the myelin sheath, MBP, and induce experimental autoimmune encephalomyelitis (EAE), GA unexpectedly inhibited EAE in both rodents and monkeys (2–5). In subsequent clinical trials, GA reduced relapse rate and progression of disability in patients with relapsing–remitting multiple sclerosis (MS) leading to its approval in 1995.

The mechanism by which GA is beneficial in central nervous system (CNS) autoimmune disease (reviewed in refs 6, 7) is thought to be mediated through a preferential T_h2 deviation of myelin-specific T cells (8–10). Other effect of GA involves modulation of CD8+ T cells (11) and antigen-presenting cells (APC) (12). Recently, GA has been shown to induce monocytes to direct differentiation of regulatory T cells (Treg) in an antigen-independent manner (13). This observation favors the hypothesis that this drug may be effective in autoimmune diseases other than MS.

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the involvement of multiple organ systems with alternating clinical exacerbations and remissions. Circulating immune complexes and auto-antibodies cause tissue damage and organ dysfunction with manifestations involving the skin, serosal surfaces, CNS and kidneys (14).

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These manifestations are believed to be the result of interactions between autoreactive lymphocytes that arise from both hereditary immunoregulatory defects and environmental factors, including chemicals and UV radiation. Autoreactive T and B cells drive the production of auto-antibodies and the formation of immune complexes, which ultimately lead to tissue damage and organ failure in SLE (15). It is well established that in SLE, several genetic factors independently contribute to the overall susceptibility and progression of the disease (16–18).

Despite many common characteristics, the mouse models of lupus exhibit unique histological and serological manifestations as well as unique disease accelerators. Male BXSB mice carry the Y-chromosome-associated accelerator of autoimmunity termed *Y-linked autoimmune acceleration* (*Yaa*). The *Yaa* mutation has been shown to be responsible for the acceleration of the lupus-like autoimmune syndrome in BXSB mice and in their F_1 hybrids with NZB or NZW mice (19, 20). More recently, the *Yaa* mutation was shown to be a consequence of a translocation from the telomeric end of the X chromosome onto the Y chromosome (21, 22). Hybrid (NZB × NZW) F1 *Yaa*-bearing mice spontaneously develop a generalized autoimmune disorder resembling human SLE (23). Monocytosis is a unique cellular abnormality associated with the *Yaa* mutation (24). In PBMCs from an 8-month-old male BXSB mouse, monocytes reach >50% of leucocytes and is thought to be a major trigger of the accelerated development of lupus disease in this animal model (25, 26). Thus, in this project, the lupus (NZB × BXSB)F1 model was chosen for its accelerated disease associated with specific monocytosis, in regard of the recent demonstration of the potential antigen-independent effect of GA on monocytes and T cell function (13).

Methods

Mice

Female NZB and male BXSB mice bearing the *Yaa* mutation were obtained from local colonies and the hybrids (NZB × BXSB)F1 male used in this study were obtained by local breeding. Mice were bled (from retro-orbital sinus puncture) at 1 and 2 months post-initiation of GA injection. C57BL/6 mice were used as negative controls for monocytosis assessment. The blood was allowed to clot at room temperature, and the serum was stored at −20°C until use.

GA treatment

GA was injected daily subcutaneous (s.c.) (150 μg in 100 μl PBS–mannitol) as previously described (13). Randomized control littermates were injected with vehicle treatment consisting of PBS and mannitol at equal concentration contained in the GA preparation. GA and vehicle treatment were injected from the age of 3 months until death. At 10 months of age, mice still alive were sacrificed and kidney histology was performed.

Serological assays

Serum levels of IgG auto-antibodies against chromatin and double-stranded DNA (dsDNA) were determined by ELISA (27, 28). Chromatin prepared from chick erythrocytes was directly coated to ELISA plates, while dsDNA was coated to ELISA plates precoated with poly-L-lysine (Sigma–Aldrich, St Louis, MO, USA). Then, the plates were incubated with 1:100 diluted serum samples, and the assay was developed with alkaline phosphatase-labeled goat anti-mouse IgG, IgG1 and IgG2a. Results are expressed in U ml^{-1} in reference to a standard curve derived from a serum pool of MRL-*Fas*^{lpr} mice.

Flow cytometric analysis of monocytosis

Flow cytometry was performed using two-color staining of PBMC and analyzed with a FACSCalibur (Becton Dickinson, Mountain View, CA, USA). The following mAbs were used: anti-CD11b (M1/70) and anti-F4/80. Mice displaying percentages of monocyte levels above the mean ± 3 SD of controls (C57BL/6) were considered as positive for monocytosis (25).

Determination of hematocrit

Blood samples were collected into heparinized microhematocrit tubes and centrifuged at 12 000 r.p.m. for 5 min in a microfuge (Sigma-201 M, Auer Bittmann Soulie AG, Geneva, Switzerland). Percentage of packed RBC volume was directly measured after centrifugation.

Detection of Coombs antibodies

A flow cytometric assay was used to detect anti-erythrocyte antibodies. After washing three times with 1% BSA–PBS, a similar number of RBC [according to their hematocrit (Hct)] was incubated with biotinylated rat anti-mouse kappa-chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin (eBioscience, San Diego, CA, USA), and analyzed by FACS. The results are expressed in ng ml^{-1} in reference to a standard curve obtained with known concentration of 34.3C IgG2a anti-RBC.

Histopathology

Kidney samples were collected when mice were moribund or at the end of the experiment (10 months of age). Histological sections were stained with periodic acid–Schiff reagent. The extent of glomerulonephritis (GN) was graded on a 0–4 scale based on the intensity and extent of histopathological changes as described previously (29). Histopathology was performed in a blinded way by expert mice lupus pathologist. GN with grade 3 or 4 was considered a significant contributor to clinical disease and/or death.

Statistical analyses

Statistical analyses were performed using PRISM software (GraphPad, San Diego, CA, USA). The statistical significance ($P < 0.05$) was determined by the non-parametric Mann–Whitney test.

Results

GA treatment does not modify mortality of (NZB × BXSB)F1 lupus mice

Mice received daily s.c. injection of GA or vehicle treatment from the age of 3 months until death. GN is a pathological

hallmark feature of murine SLE. To examine the effect of GA treatment on lupus nephritis and pathology, histological examination of the kidney was performed. Disease development started in both groups within the first month of injection. Classical progression of SLE as well as mortality was present in both GA- and vehicle-treated groups with no difference. In the GA group, median time to death was 6.0 ± 2.4 months, while in the placebo group it was 7.0 ± 2.6 months (P = ns). The Fig. 1(A) details the mortality rate of the GA- (n = 15) and the vehicle-treated (n = 17) groups. To avoid possible bias like death of mice of other cause than GN, the mortality curve includes only mice that developed typical GN with a score of 3+ or more (Fig. 1B).

GA treatment does not influence anti-erythrocytes antibody production and Hct

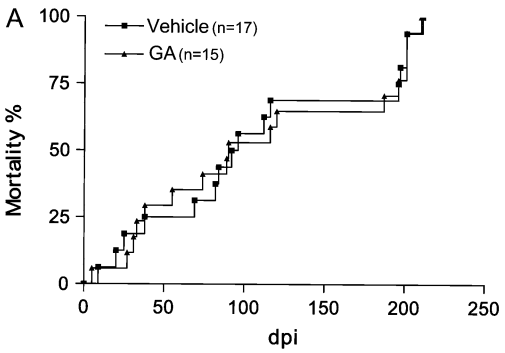
Auto-antibodies are essential factors for several clinical manifestations associated with SLE (14). The hybrid mice used in this study develop auto-antibodies directed against erythrocyte (RBC) which induce anemia that can be detected during the three to four first months of life. The production of Coombs antibodies was assessed in (NZB × BXSB)F1 male mice. No significant difference of anti-RBC antibodies was found at the age of 4 months (i.e. 1 month post-treatment injection) between GA- (n = 18) and vehicle-treated (n = 19) mice (anti-RBC ng ml^{-1} ± SEM; 0.3 ± 0.04 versus 0.45 ± 0.06; P = ns) (Fig. 2A). The anti-RBC antibodies were increased in the GA- and vehicle-treated groups when compared with 2-month-old (NZB × BXSB)F1 mice (anti-RBC ng ml^{-1} ± SEM; 0.15 ± 0.02; P < 0.05). Hct was measured in parallel to Coombs test. Hct values tested at the age of 4 months (i.e. 1 month post-treatment injection) were not different between GA- (n = 18) and vehicle-treated (n = 19) mice (Hct% ± SEM; 41.9 ± 1.2 versus 39.7 ± 1.1) but were significantly decreased as compared with those of 2-month-old mice (P < 0.05) (Fig. 2B). Anti-RBC antibodies and Hct were also measured at the age of 5 months (i.e. 2 months post-treatment injection) with no significant differences between GA- and vehicle-treated groups (data not shown).

Total IgG and subclasses IgG1 and IgG2a anti-chromatin and anti-dsDNA production are not altered by GA treatment

NZB mice are genetically predisposed to develop auto-antibodies that provoke tissue lesions as a result of their deposition as immune complexes in renal glomeruli and vessels (30, 31). To investigate whether GA treatment influences the production of auto-antibodies, total IgG, IgG1 and IgG2a subclasses against chromatin were tested in the serum of 4-month-old mice (i.e. 1 month post-treatment) by ELISA in GA- (n = 19) and vehicle-treated groups (n = 18). Compared with the vehicle-treated group, total IgG anti-chromatin titers were decreased in the GA group although a significant difference was not reached (median ± SEM; 19.1 ± 4.5 versus 37.0 ± 10.9 IgG U ml^{-1}) (Fig. 3A). Total IgG anti-chromatin from both GA- and vehicle-treated groups was significantly increased when compared with the control consisting of 2-month-old mice (before disease development) (P < 0.05). To further analyze IgG subtypes, anti-chromatin IgG1 and IgG2a were tested. IgG1 anti-chromatin titers did not show any significant difference when GA was compared with the vehicle-treated group and when both groups were compared with controls [GA versus vehicle treated versus control; 97.1 ± 20.0 versus 92.3 ± 30.6 versus 53.4 ± 6.9 U ml^{-1} (Fig. 3B)]. IgG2a anti-chromatin was significantly increased in both the GA and vehicle groups when compared with the control group (IgG2a from GA versus vehicle versus control; 12.1 ± 3.6 versus 21.2 ± 5.5 versus 2.3 ± 0.6 U ml^{-1}; GA or vehicle versus control, P < 0.05; GA versus vehicle, P = ns) (Fig. 3C). IgG, IgG1 and IgG2a against chromatin were also tested in the same groups at the age of 5 months (i.e. 2 months post-treatment initiation) with similar results (data not shown). Similar results were obtained when testing the anti-dsDNA IgG, IgG1 and IgG2a (Fig. 3D–F).

Monocytosis observed in (NZB × BXSB)F1 is not modified by GA treatment

Monocytosis is a unique cellular abnormality associated with the *Yaa* mutation (24). Monocytes reach a frequency of 50% of PBMC in 6- to 8-month-old BXSB male mice and have been associated with the rapid progression of SLE in this



B

	Glomerulonephritis grading	
	+++	++++
GA-treated (n=15)	9	6
Vehicle-treated (n=17)	14	3

Fig. 1. Mortality rate of GA- or vehicle-treated (NZB × BXSB)F1. Mice were injected at the age of 3 months with GA 150 μg s.c. or vehicle only. Injections were performed once a day until death (dpi = day post-injection). (A) No difference of mortality is observed when GA-treated group is compared with vehicle-treated group (P = ns). (B) Only mice that developed GN histology-proven graded 3 or 4 were included in the Kaplan–Meier survival curve (n = number of mice with GN histological grading 3+ or 4+).

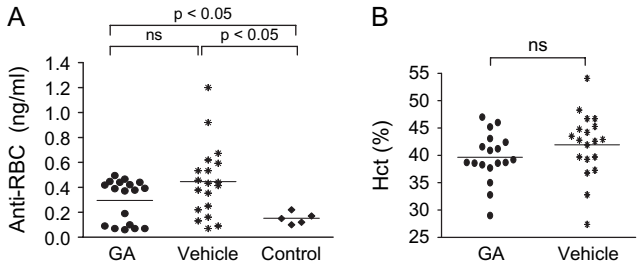


Fig. 2. Assessment of anti-erythrocyte (anti-RBC) antibodies and Hct 1 month post-treatment onset. (A) Anti-RBC antibodies were tested by the Coombs method following standard procedure. Negative control consisted in 2-month-old (NZB × BXSB)F1 mice (i.e. before disease onset). Anti-erythrocyte antibodies were significantly increased 1 month after treatment initiation in both GA- and vehicle-treated groups when compared with negative control ($P < 0.05$). When comparing GA- to vehicle-treated group, no difference was found (P = ns). Similar results were observed at month 2 post-injections (data not shown). (B) Hct was tested in both GA- and vehicle-treated groups after 1 month of treatment. Mice in both treated groups presented with Hct under 40% but no significant difference was observed between the groups.

animal model. The level of monocytosis was analyzed by FACS in both treated groups at the age of 5 and 6 months (i.e. 2 and 3 months post-treatment initiation; GA $n = 6$, vehicle treated $n = 7$). Controls consisted of 2-month-old mice of the same strain (i.e. before disease onset). The median value of monocytes expressed in % of total PBMC for GA- versus vehicle-treated mice was not different at both the second (median ± SEM; 35.4 ± 23.8% versus 35.5 ± 17.3%) and the third month post-treatment initiation (median ± SEM; 35.0 ± 10.9% versus 47.2 ± 16.8%) (Fig. 4A and B). When compared with the control group (median monocytes ± SEM; 9.1 ± 0.8%), both monocytosis of GA- and vehicle-treated groups were considerably increased at the two time points ($P < 0.05$). Monocytosis was defined as described in a previous report (25) (dotted line, Fig. 4A and B).

Discussion

Over the last three decades, despite intensive investigation of the disease and its mechanisms, there has been a lack of major improvements in the treatment of SLE. Genetic predisposition, environmental factors and complicated interactions within the immune system all contribute to the complexity of this autoimmune disease. With the characterization of several animal models, however, investigators were able to primarily focus on the mechanisms of action underlying the immunological pathophysiology. Results suggest that the autoimmune process in lupus-prone animals may be initiated by small numbers of B cells that have a low threshold for activation against self-antigens (15, 25). Therefore, it should be therapeutically beneficial to block the initial autoreactive responses as well as the activation of the downstream cascades. A unique cellular abnormality associated with the *Yaa* mutation in the lupus-prone mouse strain used in this study is an impressive monocytosis (24). The frequency of monocytes can reach over 50% of all leukocytes in 6- to 8-month-old BXSB male mice. Although the real significance of monocytosis in lupus disease remains to be determined, the development of a progressive monocytosis in this strain is associated with the rapidity of SLE development suggesting that T cells, B cells and auto-antibodies are not the unique trigger of disease progression.

Recent insights derived from studies on the mechanism of action of GA show a pivotal role of monocytes in the modulation of the immune system and highlight the importance of these cells as a target for pharmacologic intervention in autoimmune diseases (7, 12, 13). These results suggest that GA might be useful for autoimmune diseases other than MS. The pathogenesis of SLE is different from MS, although some similarities can be found. First, both diseases are considered to be triggered by a deregulation of the immune system and no definite putative antigen has been found in either situation. Both SLE and MS pathogenesis are in part driven by T cells and APC (32–34) and up to 20% of SLE have CNS involvement. In addition, anti-nuclear antibodies are characteristic for SLE and can be found in up to 30% of MS patients. Preliminary data show that rituximab, a monoclonal chimeric antibody directed against CD20 and depleting B cells, can be beneficial in both SLE and MS (35–37). A major difference between the two diseases it that SLE, more than MS, is driven by auto-antibodies and immune complexes that activate the complement pathway as confirmed by kidney histopathology. Contrary to MS, specific auto-antibodies such as anti-dsDNA can be detected in the serum of SLE patients. In MS, the pathogenesis is mostly directed by autoreactive T_h1 T cells (34), although antibody- and complement-mediated demyelination are considered as possible co-pathogenic factors in >50% of all MS patients (38).

The animal model of SLE and MS also differs in regard to their pathogenesis although both models are driven by a strong T_h1 autoimmune response. The pathogenesis of lupus-prone (NZB × BXSB)F1 mice is mainly mediated by auto-antibodies and an intrinsic B cell defect (39), whereas chronic EAE induced in C57BL/6 mice is mostly mediated by generation of encephalitogenic T_h1 and T_h17 T cells, with only a minor role for auto-antibodies associated with demyelination. NZB mice are genetically predisposed to develop Coombs-positive hemolytic anemia, splenomegaly and auto-antibodies (31). To determine the effect of GA on the progression of lupus mice's disease, we used the mouse hybrid (NZB × BXSB)F1 characterized by the strength and rapidity to develop clinical signals of SLE. In these mice, the disease onset can be present at 3 months, 50% of the population generally die within the first 5 months (40).

The hypothesis of our study was that GA, through the modulation of APC, such as monocytes and subsequently alteration of the T cell phenotype may inhibit the progression of the disease. Our data demonstrate that GA treatment initiated at disease onset (3 months of age) and continuously administered until death exerted no effect on mortality as the primary clinical readout. Further biological examinations, including standard controls of disease progression such as rise of anti-erythrocytes antibodies (Coombs test) and Hct, showed no significant difference either. Additional measurements of humoral and cellular markers of disease progression were performed. Anti-chromatin and anti-dsDNA IgG can provoke tissue lesions due to their deposition as a form of immune complex in renal glomeruli and vasculitis (14).

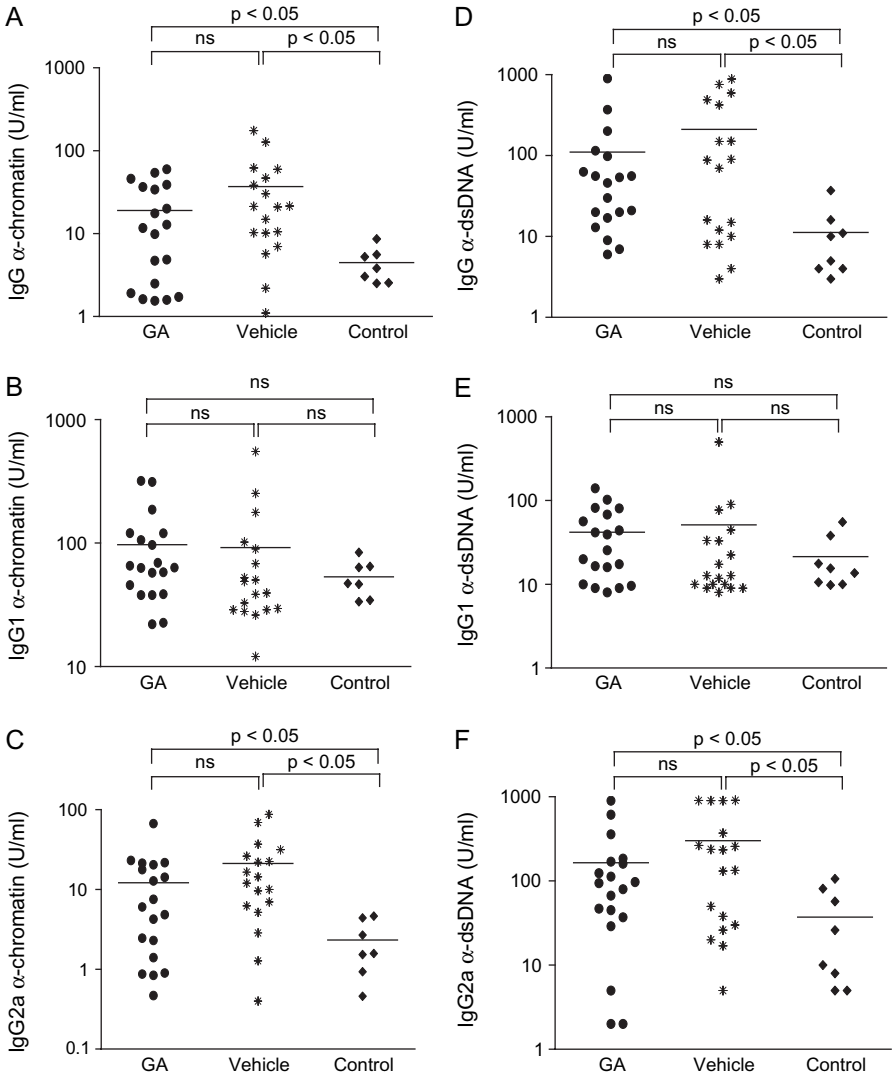


Fig. 3. Anti-chromatin and anti-dsDNA IgG subclasses examination 1 month after treatment onset. (A) Total anti-chromatin IgG is increased in the GA-treated group although significance is not reached when compared with vehicle-treated group (P = ns). Difference is significant when both treated groups are compared with control group ($P < 0.05$). (B) Anti-chromatin IgG1 subclass is similar in both treated and control groups. (C) Anti-chromatin IgG2a subclass is increased in both GA- and vehicle-treated groups but inter-group comparison is not different. Similar results were obtained when IgG, IgG1 and IgG2a against dsDNA (D–F) are tested in the serum 1 month after treatment onset. IgG, IgG1 and IgG2a against chromatin and dsDNA were also tested in the same groups 2 months post-treatment initiation with similar results (data not shown).

The influence of GA in switching and synthesis of IgG subclasses in EAE mice is unknown. In a clinical study on GA-treated MS patients, IgG1, and to a lesser extent IgG2, were found to be increased in the serum. GA is known to promote development of T_h2 cell. Thus, in our study, a GA-dependent modulation of anti-chromatin and anti-dsDNA IgG subclasses from the pathogenic IgG2a (T_h1 dependent) to the less pathogenic IgG1 (T_h2 dependent) subclass was anticipated. Total anti-chromatin and anti-dsDNA IgG, IgG1 and IgG2a subclasses were tested by ELISA 1 and 2 months post-treatment initiation. Total IgG and the predominantly pathogenic IgG2a auto-antibodies (41) increased over time in both GA- and vehicle-treated groups, with no difference between the two groups.

The progressive monocytosis observed over time in (NZB × BXSB)F1 mice was not modified in GA-treated mice as shown on Fig. 4. Recent data showed that GA could promote type II monocytes that induced Treg specific for a variety of antigens. Adoptive transfer studies demonstrated that Treg specific for a foreign antigen could ameliorate EAE, indicating that neither GA specificity nor recognition of self-antigen was required for their therapeutic effect (13). A specific Treg population was not analyzed in our study, but our results show that the frequency of monocytes was not altered by GA during lupus disease progression and that a possible effect of GA on monocytes, such as cytokine production or T cell modulation, did not modify the clinical course of the mice.

Many hypotheses can be raised about the absence of efficacy of GA in these lupus-prone mice. First, the animal model used in this study is one among several spontaneous, transgenic or toxic SLE animal models (42–44). Thus, the failure

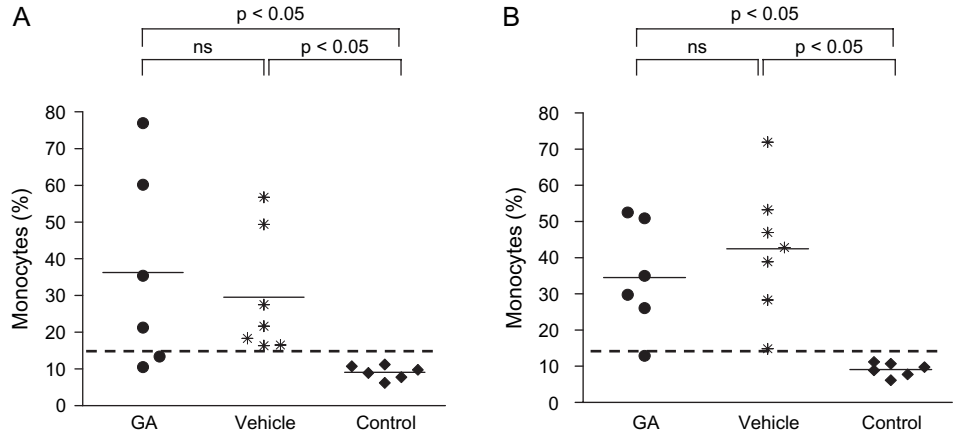


Fig. 4. Monocytosis examination in GA- and vehicle-treated mice after treatment initiation. The median value of monocytes in PBMC for GA- versus vehicle-treated mice is not different for both the second (A) and the third (B) month post-GA treatment (P = ns). Both treated groups demonstrate the presence of monocytosis when compared with control group consisting of 2-month-old (NZB × BXSB)F1 mice ($P < 0.05$). Dotted line = mean value ± 3 SD of monocytes in control group (C57BL/6 mice).

of GA treatment in preventing lupus progression in (NZB × BXSB)F1 mice cannot be taken as a general inefficacy of GA treatment in lupus animal models. Second, GA is known to induce T_h2 T cell that may potentially stimulate B cells to secrete auto-antibodies and favor SLE progression. Third, GA treatment was injected at an identical concentration as performed in EAE models and it cannot be excluded that another dose may provide different results. However, GA has been effective in the treatment of other models of autoimmune diseases including uveoretinitis (45), inflammatory bowel disease (46) and graft rejection (47). In addition to autoimmune diseases, pre-clinical data also suggest that GA may have a beneficial effect for Alzheimer's disease (48, 49). In conclusion, this is to the best of our knowledge the first report assessing the effect of GA in the treatment of a lupus animal model and results suggest, at least in the (NZB × BXSB)F1 lupus mice model, the absence of efficacy.

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Abbreviations

APC	antigen-presenting cell
CNS	central nervous system
dsDNA	double-stranded DNA
EAE	experimental autoimmune encephalomyelitis
GA	glatiramer acetate
GN	glomerulonephritis
Hct	hematocrit
MS	multiple sclerosis
s.c.	subcutaneous
SLE	systemic lupus erythematosus
Treg	regulatory T cell
Yaa	Y-linked autoimmune acceleration

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